Analysis of 25-Hydroxyvitamin D in serum by automated sample cleanup and LC-MS/MS

MACHEREY-NAGEL application department
Dr. H. R. Wollseifen, Dr. A. von Bohl, T. Kretschmer, L. Schaefer

Application benefits

- Automated sample cleanup of 25-Hydroxyvitamin D in serum
- Analysis without time-consuming, costly evaporation and reconstitution steps
- Facilitates high sample throughput utilizing automation in a multi-well plate format

MN products

REF 763632.20
EC HPLC column (analytical), NUCLEOSHELL® Biphenyl, 2.7 µm, 50x2 mm

REF 738921.010M
SPE MULTI 96-well plate CHROMABOND® HLB, 96x 10 mg, 30 µm

REF 702107
Screw closure, N 9, PP, yellow, center hole, Silicone white/PTFE red, 1.0 mm

REF 702079
Screw neck vial, N 9, 11.6x32.0 mm, 1.5 mL, label, flat bottom, amber, silanized

Eppendorf product

epMotion® 5075vt –
Catalog no. 5075000044
(www.eppendorf.com/epMotion5075)

MN application numbers

SPE: 306990
HPLC: 129580

Keywords

25-Hydroxyvitamin D2, 25-Hydroxyvitamin D3, serum, automated analysis, SPE MULTI 96-well plate CHROMABOND® HLB, NUCLEOSHELL® Biphenyl

Introduction

Vitamin D is the generic term for a group of fat-soluble vitamins, the so-called calciferols, which belong to the secosteroids. The most important forms include vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol). They are mainly known for their function in calcium metabolism and therefore play a key role in bone mineralization. Additionally evidence of a link between vitamin D supply and a variety of diseases (hypertension, type 2 diabetes mellitus, and cardiovascular and cancer diseases) was found [1].

The determination of vitamin D level is therefore increasingly necessary and is carried out by measuring 25-Hydroxyvitamin D, 25-OH Vit Dx (X = 2 and 3), in blood serum. In recent years, liquid chromatography combined with mass spectrometry (LC-MS/MS) has become established for the measurement of 25-OH Vit D in serum [2]. In order to ensure the highest possible sample throughput, an automated SPE methodology using a 96-well plate, packed with CHROMABOND® HLB sorbent, was developed in this application note. Automated solid phase extraction procedure was performed using the epMotion® 5075vt robot.

High recovery rates with very good reproducibility are achieved for serum samples. Finally, the extracts are analyzed using HPLC-MS/MS on a NUCLEOSHELL® Biphenyl column.

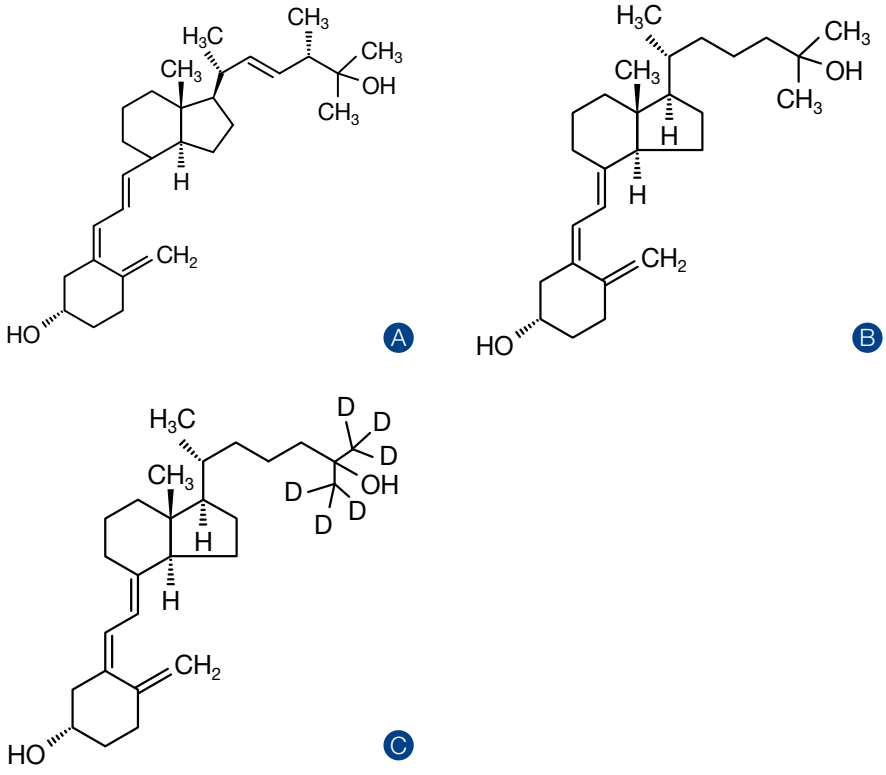


Figure 1: Molecular formula of 25-Hydroxyvitamin D (A: 25-hydroxyvitamin D2, B: 25-hydroxyvitamin D3, C: d6–25-hydroxyvitamin D3)

Analysis of 25-Hydroxyvitamin D in serum by automated sample cleanup and LC-MS/MS

Sample pretreatment

MN Appl. No. 306990

Sample preparation:

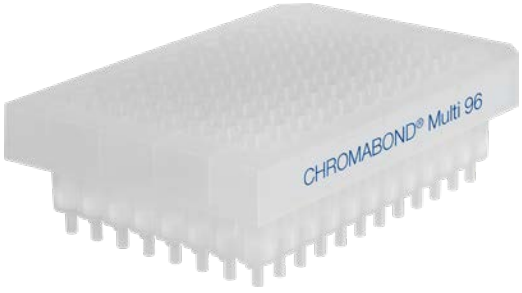
1. Put 150 µL of serum sample in 96-deep-well plate.
2. Add 20 µL internal standard-solution (β = 250 ng/mL in methanol).
3. Add 150 µL aqueous zinc sulphate solution (0.2 M).
4. Add standard-solution (x µL in methanol*).
5. Add 600 µL (- x µL standard-solution**) of methanol.
6. Centrifugate for 5 min, 750 x g.

Procedure of the addition of standard solution before precipitation of serum

Concentration sample solution (ng/mL)	* Volume of standard solution (µL)	Concentration standard solution (ng/mL)	** Volume of methanol (µL)
0	0	0	580
2	28.1	8	552
6	28.1	32	552
9	28.1	48	552
21	28.1	112	552
51	28.1	272	552
99	28.1	528	552
201	28.1	1072	552
300	28.1	1600	552

Automated solid phase extraction procedure on the epMotion® 5075vt robot

Parameter	Description
Column	SPE MULTI 96-well plate CHROMABOND® HLB, 96x 10 mg, 30 µm
Conditioning	500 µL methanol, vacuum pressure
Loading	600 µL pretreated sample (low vacuum pressure, e. g. -100–200mbar)
Wash 1	500 µL 5 % methanol (aq); vacuum pressure
Wash 2	500 µL 60 % methanol (aq); vacuum pressure
Drying	/
Elution 1	240 µL 95:5 (v:v) methanol/ isopropanol
Elution 2	150 µL LC-MS-water



Analysis of 25-Hydroxyvitamin D in serum by automated sample cleanup and LC-MS/MS

Analysis by HPLC-MS / MS

MN Appl. No. 129580

Chromatographic conditions

Column	NUCLEOSHELL® Biphenyl, 2.7 µm, 50x2 mm
Eluent A	0.1 % formic acid in water
Eluent B	0.1 % formic acid in acetonitrile
Gradient	Time % B 0 min 65 2 min 65 3 min 95 6 min 95 7 min 65 10 min 65
Flow rate	0.4 mL/min
Temperature	40 °C
Injection volume	5 µL
MS conditions for Shimadzu 8050 – Triple Quadrupole MS	
Acquisition mode	MRM
Interface	ESI
Polarity	positive
Nebulizing gas flow	3 L/min
Heating gas flow	10 L/min
Interface temperature	300 °C
DL Temperature	250 °C
Heat Block Temperature	400 °C
Drying gas Flow	10 L/min
CID gas	270 kPa Argon

MRM transitions

Analyte	Abbreviation	CAS number	Q1 mass [Da]	Q3 mass [Da]	Q3 mass [Da]	Q3 mass [Da]	Retention time [min]
25-hydroxyvitamin D2	25-OH Vit D2	21343-40-8	395.20	105.10	135.30	137.10	4.26
25-hydroxyvitamin D3	25-OH Vit D3	140710-94-7	383.15	365.30	120.90	271.30	3.96
d6–25-hydroxyvitamin D3	d6–25-OH Vit D3	78782-98-6	389.20	371.25	133.30	45.10	3.92

Table 2: MRM transitions and retention times of 25-hydroxyvitamin D.

Chromatogramms

Figure 2: a

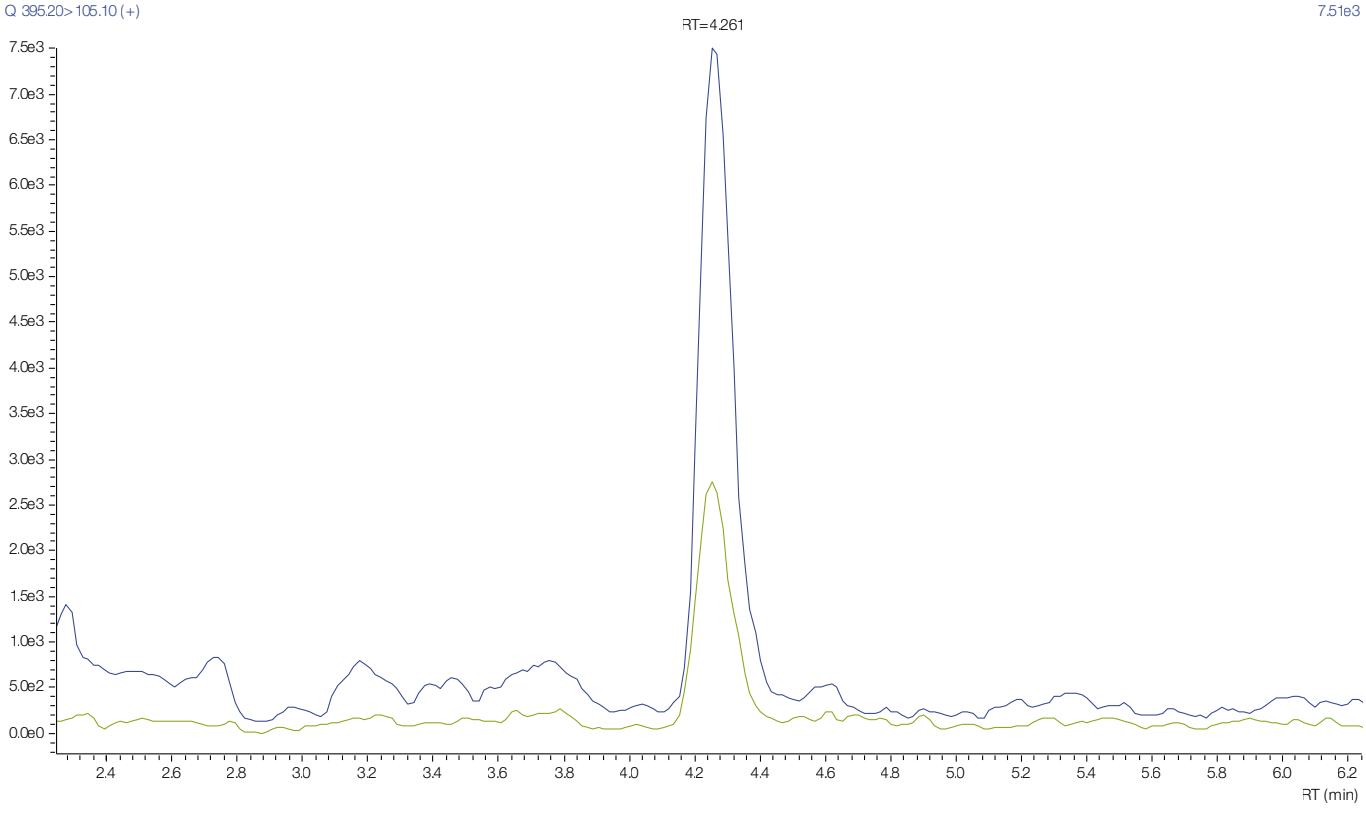


Figure 2: b

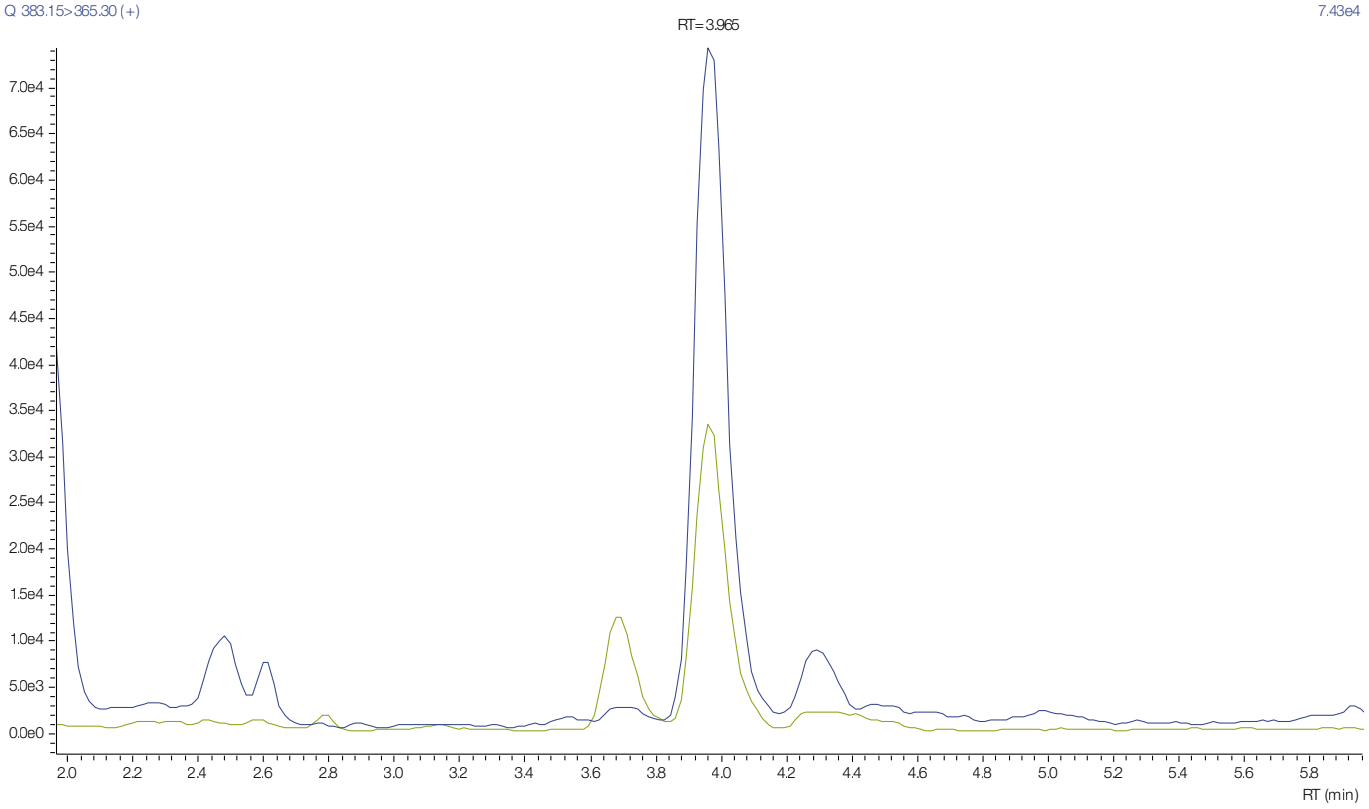


Figure 2: c

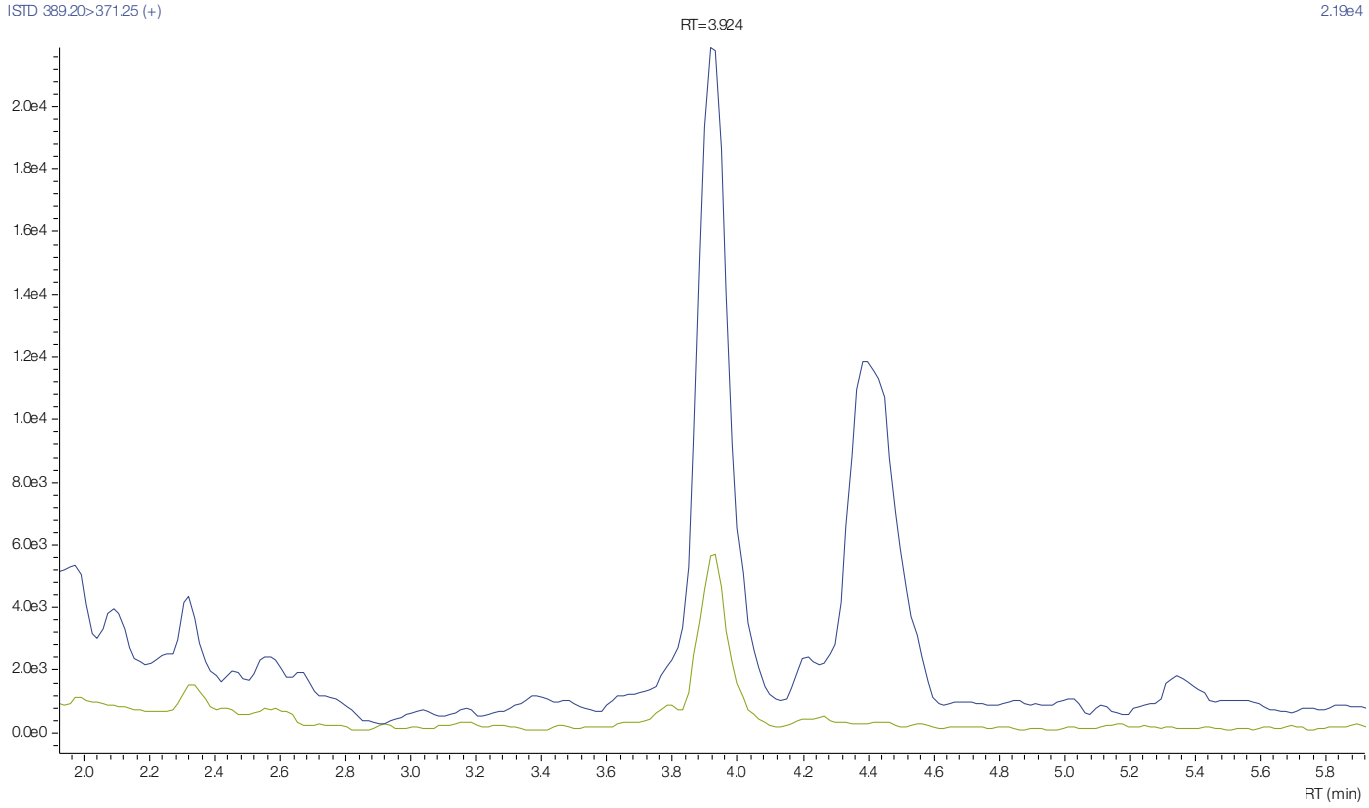


Figure 2: Chromatograms of a matrix matched standard solutions (concentration β = 21.0 ng/mL, A: 25-OH Vit D2, B: 25-OH Vit D3, C: d6–25-OH Vit D3)

Calibration curves

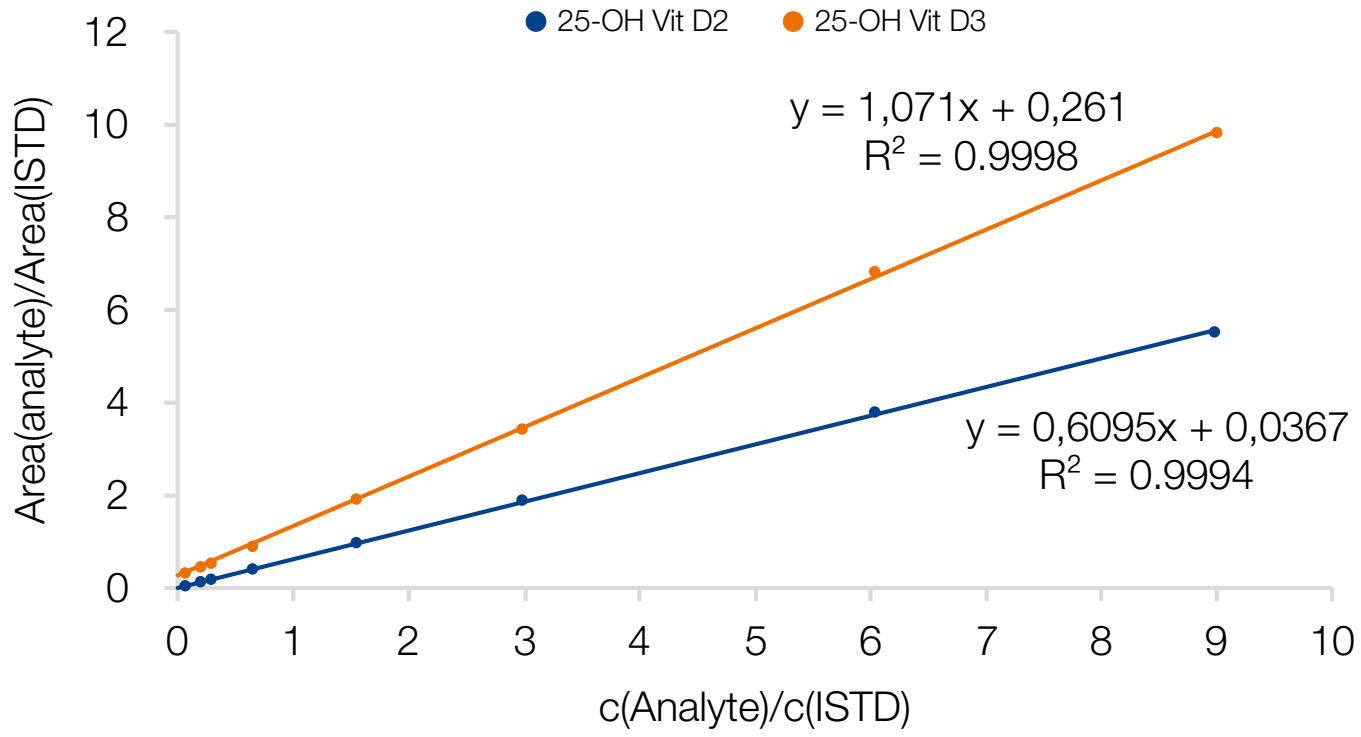


Figure 3: The matrix matched calibration range for 25-OH Vit D2 and 25-OH Vit D3 between 1.5 – 301.0 ng/mL

Recovery rates

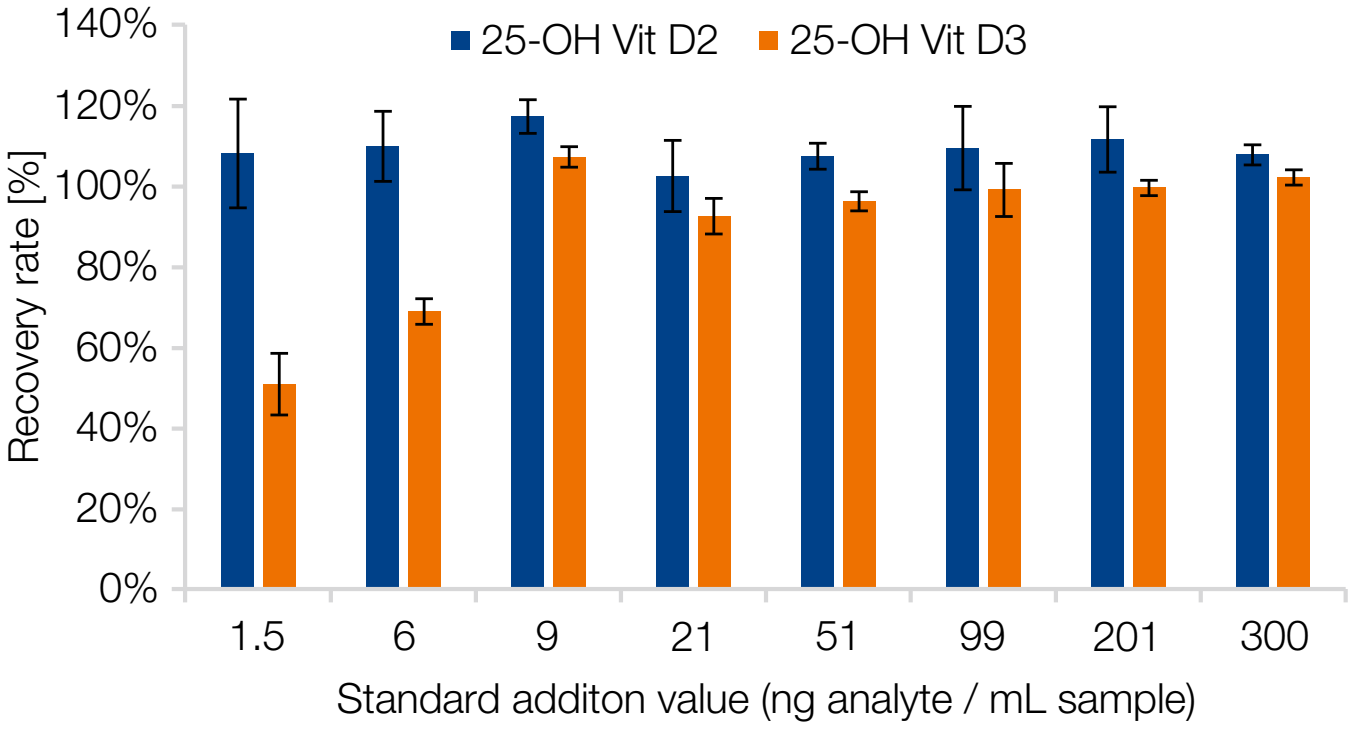


Figure 4: Recovery rates for the presented automated SPE method using SPE MULTI 96-well plate CHROMABOND® HLB, 96x 10 mg, 30 µm.

Analysis of 25-Hydroxyvitamin D in serum by automated sample cleanup and LC-MS/MS

Standard addition concentration (ng/mL)	Recovery rate [%] 25-OH Vit D2	Recovery rate [%] 25-OH Vit D3
1.5	108 % ± 13 %	51 % ± 8 %
6	110 % ± 9 %	69 % ± 3 %
9	117 % ± 4 %	107 % ± 3 %
21	103 % ± 9 %	93 % ± 4 %
51	108 % ± 3 %	96 % ± 2 %
99	110 % ± 10 %	99 % ± 7 %
201	112 % ± 8 %	100 % ± 2 %
300	108 % ± 2 %	102 % ± 2 %

Recovery rates for the presented automated SPE method using SPE MULTI 96-well plate CHROMABOND® HLB, 96x 10 mg, 30 µm.

Conclusion

This application note presents a reliable and successful determination of 25-Hydroxyvitamin D2 and 25-Hydroxyvitamin D3 in serum. By using a SPE MULTI 96-well plate CHROMABOND® HLB (96x 10 mg, 30 µm) on the epMotion® 5075vt robot, it was possible to achieve high recovery rates and good reproducibility. As a result for the tested standard addition values, most recovery rates are between 90 % and 110 %. Furthermore, the presented SPE methodology eliminates the need for time-consuming solvent evaporation and reconstitution steps.

The identification and the quantification of 25-Hydroxyvitamin D in serum was finally carried out by ESI mass spectrometry on a NUCLEOSHELL® column. Using core-shell particle technology allows, highest column efficiency and resolution at a short run time with much lower back pressure compared to fully porous particles could be achieved with common HPLC systems. The Biphenyl modification successfully helps to separate analytes from matrix with different retention mechanisms: π-π interactions and hydrophobic interactions.

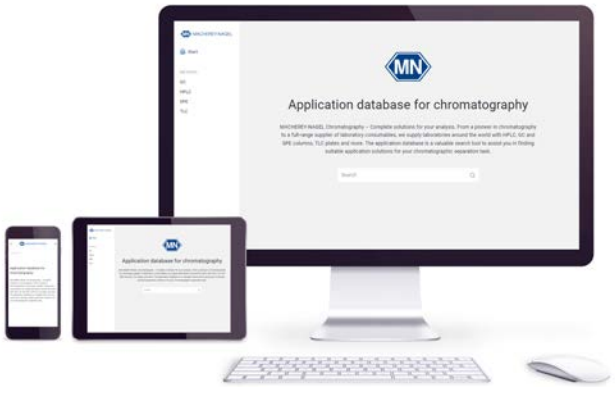
References

[1] Teresa Kulie et al, Vitamin D: An Evidence-Based Review, JABFM, 2009, vol. 22 no 6.

[2] Kornelia Galior, Hemamalini Ketha, Stefan Grebe, Ravinder J Singh: 10 years of 25-hydroxyvitamin-D testing by LC-MS/MS-trends in vitamin-D deficiency and sufficiency Bone Reports (2018), 268–273

The chromatography application database

- Free access to more than 3,000 application examples from HPLC, GC, TLC and SPE
- Free access to the MN application database: *https://chromaappdb.mn-net.com*



www.mn-net.com

MACHEREY-NAGEL

MACHEREY-NAGEL GmbH & Co. KG · Valencienner Str. 11 · 52355 Düren · Germany
DE +49 24 21 969-0 info@mn-net.com
CH +41 62 388 55 00 sales-ch@mn-net.com
FR +33 388 68 22 68 sales-fr@mn-net.com
US +1 888 321 62 24 sales-us@mn-net.com